Changes in Polyphenols in 'Rio Red' Grapefruit Leaves in Response to *Elsinoë australis* Infection

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Abstract Sweet orange scab (SOS) is a fungal disease of citrus which is caused by *Elsinoë australis*. It affects the aesthetics of the fruit by forming wart-like protruded lesions on the fruit skin, and also affects the leaves which act as source of inoculum in the orchards. SOS is widespread in the different citrus species such as grapefruit (*Citrus* x *paradisi* Macfd.), sweet orange [*C. sinensis* (L.) Osb.], and mandarin (*C. reticulata* Blanco). In this study, we report the development of symptoms and changes in polyphenolic profile of 'Rio Red' grapefruit leaves in response to *E. australis* infection. Qualitative and quantitative analysis of polyphenols in 'Rio Red' grapefruit leaves inoculated with *E. australis* were conducted using high performance liquid chromatography (HPLC) at two different time points post inoculation. Development of SOS symptoms under light was recorded and categorized into four different morphological stages. Caffeic acid, luteolin-7-O glycoside, naringin, naringenin, apigenin-7-O glycoside and eriodictoyl were identified in healthy grapefruit leaves. The quantitative changes in the identified polyphenols were measured 9 days post-inoculation by comparing levels on the 9-day control versus the 9-day post-inoculated leaves. Total polyphenol levels decreased substantially in *E. australis*-infected 'Rio Red' grapefruit leaves 9 days post-inoculation. Levels of naringin, naringenin, apigenin glucoside and eriodictoyl decreased with *E. australis* infections indicating the pathogen's ability to overcome some of plant's defenses. Compared to 9-day controls, 9-day inoculated leaves had much less caffeic acid.

Keywords Citrus · HPLC · grapefruit · sweet orange scab

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Introduction

Sweet orange scab (SOS) disease with a new scab disease symptomology in the United States was first reported by Kunta *et al.* (2011, 2013). The disease is caused by a fungal pathogen *E. australis,* and is marked by wart-like raised pustules on the citrus fruits (Bitancourt 1937). According to Bitancourt

(1937) the disease in South America is prevalent only in sweet oranges and tangerines, but recent surveys in Texas by USDA-APHIS-PPQ and Texas A&M University-Kingsville, Citrus Center in Weslaco have found it to occur in several citrus cultivars (Kunta *et al*. 2011, 2013). The characteristic raised red colored pustules on the fruit surface due to SOS drastically reduce the marketability of fresh fruit. Since grapefruit is an important commodity in South Texas (Sauls 2008), losses in grapefruit industry due to SOS are economically significant. Symptoms of SOS have morphological similarities to common scab caused by *Elsinoë fawcettii* which also occurs in various citrus cultivars (Bitancourt 1937; Jenkins 1936; Timmer 1974). Molecular identification of *Elsinoë* spp. using specific primers has been developed (Hyun *et al*. 2007; Tan *et al*. 1996).

The interactions between plants and pathogens can lead to changes in the plant's secondary metabolism. It is well known that plant pathogens and pest infestation induce changes in secondary metabolites such as polyphenols that may be involved in plant defense against the pathogen invasion (Nicholson and Hammerschmidt 1992). Changes in the level of polyphenols depend upon whether the cultivar exhibits susceptible, hypersensitive or resistant reactions (Kosuge 1969; Lattanzio *et al*. 2006). Plants fight pest or pathogen attacks using different strategies. The oxidation products of phenolic compounds are toxic and adversely affect pathogen development, and in some cases, esterification of polyphenol or lignification of plant cell walls produces physical barriers to the invasion of the pathogen (Kosuge 1969). Accumulations of phenolic acids have been observed as a first response of the host to fungal pathogen invasion at the infected sites in *Venturia inequalis*- infected apple (Mayr *et al*. 1997; Mikulič Petkovšek *et al*. 2008, 2009). Antifungal activity of phenolic compounds has been reported in olives against *Phytophthora* spp. by inhibiting mycelial growth *in vitro* in olives (Del Río *et al*. 2003). In citrus, the role of phenolic compounds is not limited to characteristics of aroma and flavor. In fact, changes in the level of polyphenols are recorded in many plant-pathogen interactions. For example, green mold-infected sweet orange fruits showed reduced levels of naringin while *Phytophthora citrophthora* infection in Valencia orange fruits produced changes in polymethoxyflavones and flavanones contents (Del Río *et al*. 2004; Ortuño *et al*. 2006). To quantify the changes in the plant metabolites

including polyphenols, High Pressure Liquid Chromatography (HPLC) technique is widely used.

Considering reduction in fresh fruit value of grapefruit in market due to SOS and the involvement of polyphenols in plant's defense mechanism, the objective of this study is to quantify changes in the polyphenol levels in response to *E. australis* infection in 'Rio Red' grapefruit leaves. The information obtained in our study may help to study the role and mechanism of different polyphenols in the plant-pathogen interactions.

Methods and Materials

Experiment set up to investigate changes in polyphenols

Fungal cultures

The *E. australis* isolate (isolate number SOS019) that was obtained from sweet orange in a previous study (Kunta *et al*. 2013) was used for conidia production and detached leaf inoculations. Genomic DNA was extracted from 100 mg fungal mycelia using Qiagen DNeasy plant mini kit (Qiagen Inc., Valencia, CA) following manufacturer's recommendations. To confirm the isolates are *E. australis*, PCR amplification was performed on 2 μL of genomic DNA in a 25 μL reaction using Platinum® Taq DNA Polymerase (Life Technologies, Carlsbad, CA) and *E. australis* species specific primers as previously described by Hyun *et al*. (2007).

Plant material preparation, and detached leaf assay

Young grapefruit (*C. paradisi* cv. 'Rio Red') leaves were collected from a flush obtained after pruning of the trees at Texas A&M University-Kingsville Citrus Center in Weslaco, TX. Leaves collected were about 4-5 cm long. Leaves were disinfected with 25% of sodium hypochlorite for 30- 45 s followed by 50% alcohol rinse for one minute and rinsed serially three times with autoclaved water. Leaves were blot dried with sterilized filter paper and placed on a strip of sterilized cheese cloth facing abaxial side up in the petri-dish. Leaves were individually inoculated with 5 μL (conidial concentration ~1.1 x 10^5/10 μL) of inoculum of *E. australis* at one spot on upper right hand corner. Conidia were obtained by following the protocol of spore production as previously described (Kunta

et al. 2013). In addition to inoculated leaves, each time a set of leaves was inoculated with sterile water drop, and treated as un-inoculated control leaves. Petri plates were sealed with Parafilm® and kept 24 h under light at 25 °C ± 1 °C. The appearance of disease symptoms was recorded from day 0 through day 25. A total of 30 each of inoculated and un-inoculated young leaves with three replications of 10 leaves in each replication were used in HPLC analysis. From each leaf, a tiny piece of 1 cm diameter tissue was collected from around the *E. australis*-inoculated and un-inoculated spot at a given time interval. The leaves were sampled at day 0 and 9 day after symptom development.

Scoring

Symptoms of disease were scored from 0 (no change) to stage 4 (fully developed lesions). Lesions at different stages of development (*i.e.* 0, 1, 2, 3 and 4) were observed by using the Dino-Lite digital microscope (Dino-Lite, Torrance, CA).

The sampling method of leaf tissue for extraction and analysis of polyphenols

For polyphenol extraction and analyses, one cm strip of leaf tissue, at least 5 mm from the visible lesions were used. Infected leaves were sampled at 0 and 9 days after inoculation (DAI). Un-inoculated leaves were also sampled at the same time.

Extraction of polyphenols

Polyphenols were extracted following previously published standard procedures (Malik and Bradford 2006). Collected samples were frozen in the liquid nitrogen and stored in -80°C until used for the extraction. For extraction, about 100 mg of leaf powder was homogenized in 1 ml of 80% methanol with Polytron™ PT 1300D Homogenizers (Kinematica™, Lucerne, Switzerland) for 30 s. The homogenization procedure was repeated twice at 10 seconds interval and the slurry was then centrifuged at 25,000 rpm for 25 minutes at 9°C using a benchtop Eppendorf refrigerated centrifuge (Eppendorf, Westbury, NY). The supernatant liquid was decanted into three separate 2 mL microfuge tubes and the pellet was re-extracted two more times with 1 mL of 80% methanol. The supernatant liquids from the three

extractions were pooled together and reduced to dryness with SPD 1010 Speedvac® system (Thermo Savant, Holbrook, NY). The dried samples were reconstituted in 80% methanol to a final volume of 1 mL. The extract was finally filtered through 0.22 μm Costar® Spin-X® filter tube filters (Corning. Inc, Tewksbury, MA). The filtrate was stored at -80 °C until used for HPLC analysis. Each leaf sample was extracted in triplicate and each extract was analyzed by HPLC in triplicates as described previously (Malik and Bradford 2006; Malik *et al.* 2009).

HPLC analysis of polyphenols

Phenolic compounds were analyzed in a WATERS 2996 HPLC (Waters, Milford, MA) system with a photo diode array detector at 280 nm ultra-violet (UV) wavelength using a WATERS XTerra MS C_{18} column measuring 5 μm particle size and dimensions are 3.9 mm x 150 mm (Waters, Milford, MA) maintained at 35°C, and eluted at 1 mL/min as described previously. The individual phenolic compounds were detected by comparing them with retention times and UV spectra of respective standards. The samples were run for 40 min with 10 μL sample per injection. Concentrations of compounds were calculated from the chromatogram by using a standard equation and expressed as μg/100 mg.

Determination of total polyphenols

A reconstituted extract from each leaf sample was used to determine total phenolic content. A 100 μL aliquot of each sample extract was diluted in the deionized water to make final volume up to 1,000 μL to prepare a 10 fold dilution. The diluted sample was then mixed with 100 μL of deionized water to make final volume 200 μL. One tenth-fold diluted Folin-Ciocalteau (FC) reagent was added to 200 μL of the previously diluted extract and then mixed with 800 μL of 7.5% of sodium carbonate solution. The mixture of diluted extract, FC reagent and sodium carbonate were vortexed thoroughly and incubated at room temperature for 30 min. After incubation of the extract-folin reagent, total polyphenols were determined by measuring absorbance at 765 nm and comparing the readings with regression curve of standard

compound (Gallic acid) as described previously (Malik and Bradford 2006; Malik et al. 2009).

Data analysis

The data were analyzed using InStat® software, version 3.0 (GraphPad Software Inc, La Jolla, CA). The significant differences between the levels of individual and total polyphenol in *E. australis*-infected leaves were tested using one-way-analysis (ANOVA) with Student-Newman-Keuls test at a significant level of 0.001. Significant differences were also calculated to see changes in the levels of polyphenols in inoculated and control leaves on the day (0 and 9) basis. Significant development of the SOS symptoms was determined using analysis of variance in 9.1 SAS at 5% (SAS institute, Cary, NC).

Results

Confirmation of *E. australis* isolate

E. australis isolate (isolate number SOS 019) from our previous study (Kunta et al. 2013) was used in leaf and fruit inoculations. The PCR amplification on fungal DNA using *E. australis* species specific primers Eaut-5 (Hyun et al. 2007) resulted in 363 base pair amplification products of expected size (results not shown).

Microscopic observations of progress of SOS symptoms

Development of the SOS symptoms was observed and categorized into four stages based on significant ($P<0.05$) morphological differences observed (Fig. 1). The first stage was observed when callus-like masses appeared at the inoculated spot on the leaves under light condition, on average by the end of 4th day. The next symptom development was the appearance of brown colored flakes, and considered as the second stage. It was observed under the microscope about 5 to 8 DAI (Fig. 2). The third stage was marked by the dark red colored flakes that were visible to the naked eyes, and observed about 11 DAI. The fourth stage which was considered as the last stage in which tissue necrosis of the infected area occurred with very short length mycelia starting to become visible. This stage was observed 15-17 DAI. The progress of symptoms was observed up to 17-19 DAI, after which necrosis and degradation of the tissue occurred.

Identification of Polyphenols

The polyphenol profile of healthy 'Rio Red' grapefruit leaves were identified based on retention time and ultraviolet spectrum using HPLC. It showed the caffeic acid, luteolin-7-O-glucoside, naringin, apigenin-7-O-glucoside, eriodictoyl, naringenin at 9.4, 21.7, 23.7, 24.1, 27.7, 32.08 retention times respectively (Fig. 3).

Fig. 1 Four stages observed *in vitro* in the sweet orange scab symptom development. **a** callus-like structures at the first stage of the infection; **b** brown flakes during the second stage; **c** scattered red spots of infection at the third stage and **d** necrosis of inoculated leaf area during the fourth stage

Symptoms Development
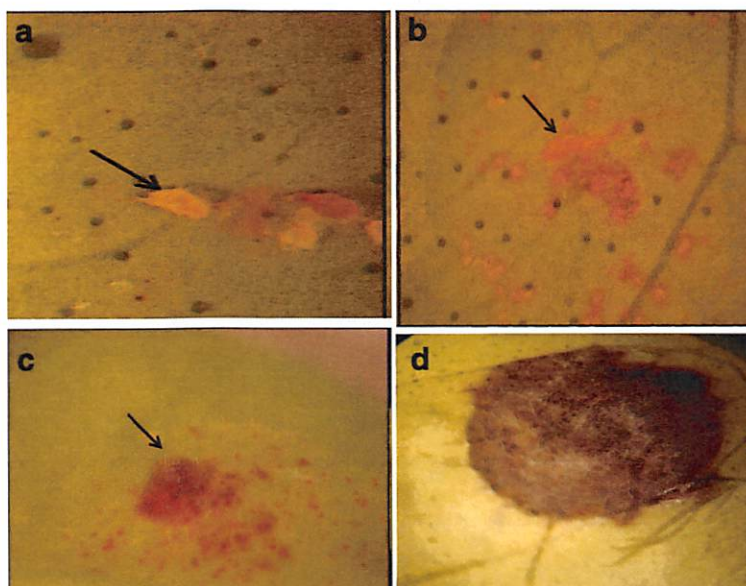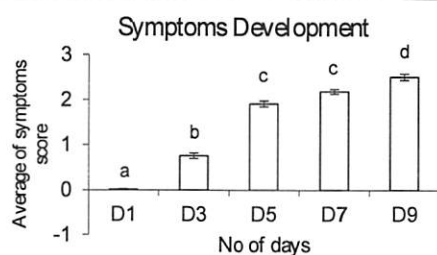
Fig. 2 Advancement of sweet orange scab symptoms on 'Rio Red' grapefruit leaves inoculated with *Elsinöe australis* from day 1 to day 9 under 24 h light. Letters indicate the significant changes in the symptom morphology through day 1 to day 9. 'D': Number of days after inoculation

Total Polyphenol content

Fig. 4 Changes in total polyphenol concentration in grapefruit leaves under light. Bars with different letters show the quantitative differences in total polyphenols in un-inoculated control leaves at day 0 and day 9. Numbers represent the significant differences in the total polyphenol concentrations in *Elsinöe australis*-inoculated grapefruit leaves on day 0 and day 9 at $P < 0.001$

Quantitative differences were observed for the same polyphenols in *E. australis* infected and control grapefruit leaves through the course of developing symptoms at stage 2 which were recorded on leaves at 9 DAI under light conditions.
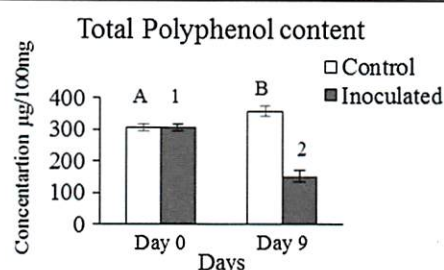
Changes in Total Polyphenols

Total polyphenol levels increased significantly (P <0.001) under light conditions in the un-inoculated leaves at the end of day 9. This increase was 49% compared to day 0 leaf samples.

E. australis-inoculated leaves showed a 51% decrease in the levels of total polyphenol at the end of day 9 when compared to day 0 inoculated samples (Fig. 4).

Changes in individual polyphenols under light

Caffeic acid level increased significantly in the un-inoculated control whereas in inoculated 'Rio Red'

leaves it decreased at the end of day 9. This increase was 32% of the initial level on day 0 in un-inoculated plant and 31% decrease of the day 0 level in *E. australis*-inoculated leaves (Fig. 5a). In the light, luteolin-7-O-glucoside level changed; however, it was not significantly affected by the *E. australis* infection throughout the 9 days, whereas it had significantly increased in un-inoculated leaves. This increase was twice as much the day 0 level (Fig. 5b).

Naringin levels increased by 18% at the end of day 9 compared to the day 0 level in the control 'Rio Red' leaves, but this increase was not statistically significant (Fig. 5c). In inoculated leaves, it decreased substantially to 36.9% compared to its initial level on day 0 (Fig. 5c). Naringenin levels at the end of day 9 in the un-inoculated and inoculated leaves showed a similar pattern to naringin. Un-inoculated leaves were found to have 64.8% notable increase, and inoculated leaves showed 58% decrease in the level of naringenin at the end of day 9 compared to the
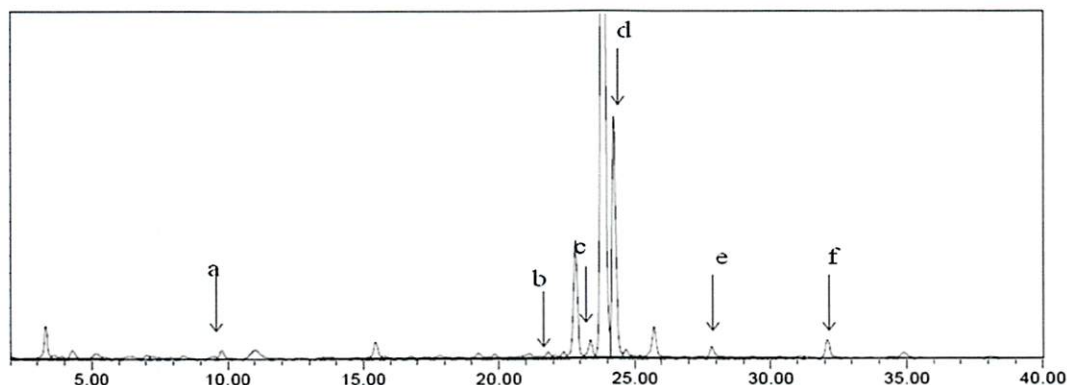
Fig. 3 HPLC Chromatogram showing polyphenols profile of 'Rio Red' grapefruit un-inoculated leaves. **a** caffeic acid at 9.4 (min), **b** luteolin-7-O-glucoside 21.7, **c** naringin 23.7, **d** apigenin-7-O-glucoside at 24.1, **e** eriodictoyl 27.7 and **f** naringenin 32.08
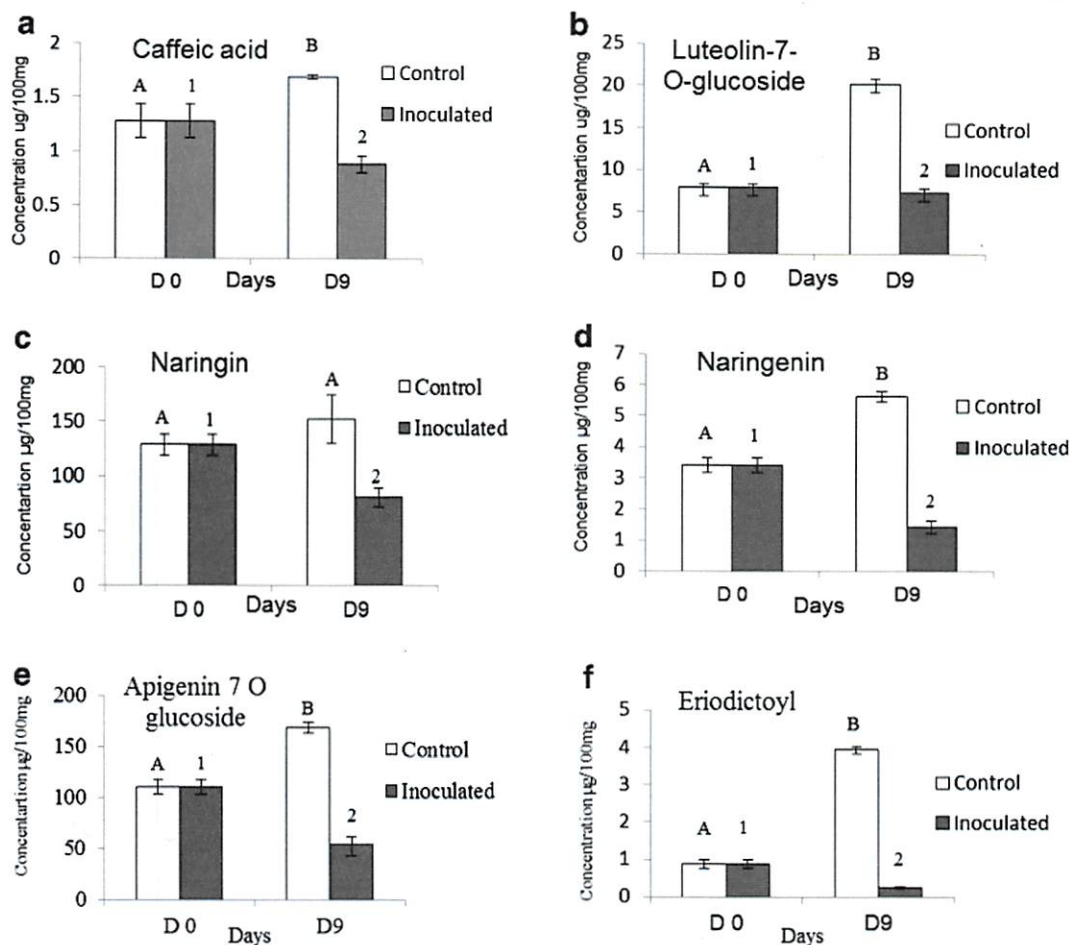
Fig. 5 Changes in the concentration of individual polyphenol levels in grapefruit leaves inoculated with *Elsinöe australis* and un-inoculated control leaves under light conditions **a** Caffeic acid, **b** luteolin-7-O-glucoside, **c** Naringin, **d** Naringenin, **e** Apigenin-7 O glucoside, **f** Eriodictoyl. Bars with different letters show the quantitative differences in individual polyphenol between control treatment leaves on day 0 and day 9. Numbers represent the significant differences in the concentration of inoculated grapefruit leaves on day 0 and day 9 at $P < 0.001$

their day 0 level (Fig. 5d). Apigenin-7-O-glucoside in un-inoculated leaves showed the opposite trend to that of inoculated leaves when compared to their initial levels at day 0. In inoculated leaves, the level of apigenin-7-O glucoside decreased by 51% and in un-inoculated leaves, it increased by 52.83% by the end of day 9 (Fig. 5e). These changes at the end of day 9 were significantly different to those of the day 0 level. In the light, eriodictoyl concentration at the end of day 9 was significantly lowered compared to their day 0 concentrations in the inoculated 'Rio Red' grapefruit leaves (Fig. 5f). This decrease in concentration was 71.6%. Un-inoculated leaves also showed significant decrease in the eriodictoyl concentration compared to their day 0 concentrations.

Discussion

The *E. australis* fungal cultures that were used to produce conidia for this study were obtained from our previous study (Kunta *et al.* 2013). They were confirmed to be *E. australis* by performing PCR amplification on fungal DNA using species specific primers (Hyun *et al.* 2007). The progression of the symptoms on inoculated 'Rio Red' grapefruit leaves was observed under a portable digital microscope and the symptom development was categorized into four different stages.

Polyphenols chosen in this study tend to fluctuate in levels in 'Rio Red' grapefruit leaves in response to the *E. australis* infections. The levels of all six major polyphenols in grapefruit leaves were lower in infected

leaves compared to un-inoculated control leaves 9 days post inoculation (Fig. 5). This suggests that all of the six identified polyphenols are probably associated with defense mechanism in grapefruit and that progress of *E. australis* infections were able to overcome these defenses by reducing the amounts of each of the polyphenol relative to their un-inoculated controls. Initial response of plants to the pathogen attack involves changes in programmed cell death (PCD), callose formation, induction or destruction of secondary metabolite, hormonal changes such salicylic acid induction etc. (Jones and Dangl 2006). Previous studies on metabolomics analysis of plant-pathogen interactions have reported the shift in polyphenol profiles as a front line of defense to the pathogen attack (Del Río *et al.* 2003, 2004; Ortuño *et al.* 2006). While high levels of certain polyphenols could deter pathogenic attack, in other cases pathogens could overcome such defense mechanism by either by destroying or modifying defense compounds or by producing their own toxin or both. For example, elsinochrome is a fungal toxin reported to produce by *Elsinoë* spp. and was studied as a virulence factor necessary for the necrosis of plant tissue (Liao and Chung 2008). Other examples where pathogen was able to overpower the defense response from the specific polyphenol similar to our results have been reported (Lattanzio *et al.* 2006; Nicholson and Hammerschmidt 1992).

In this study, light seemed to increase the levels of different polyphenols which is expected because it is well known that light delays senescence in detached leaves (Thimann 1978). In addition the effect of light irradiance on phenolic compounds has been shown in different plants (Forrest 1969; Shoji *et al.* 2011). However, in all cases the positive effect of light on polyphenols is negated by the *E. australis* infection. Thus post-infection levels of naringin, naringenin decreased significantly compared to their un-inoculated controls under light condition, indicating specific relation of these compounds to *E. australis* infection to 'Rio Red' grapefruit leaves. The findings of decrease in all of polyphenols compared to their un-inoculated controls with advancement in the SOS symptoms is consistent with the concept that the higher level of polyphenols generally acts as first line of defense and hence their decline should be related to increased susceptibility (Báidez *et al.* 2007; Del Río *et al.* 2004; Kosuge 1969; Lattanzio *et al.* 2006). Also, a decrease in phenolic compounds is observed in susceptible varieties while increases in levels are observed in resistant varieties (Reid *et al.* 1992).

Decreases in glycosylated polyphenols such as apigenin-7-O-glucoside may also relate to the possibility that pathogens might overproduce hydrolytic enzymes to simply cleave glycoside moiety and/or mobilize them into different forms (Del Río *et al.* 2004). Additional metabolomic studies are therefore needed to investigate biochemical changes such as change in the level of hydrolytic enzymes with the progression of the SOS disease for greater understanding of plant-*E australis* interaction.

In conclusion, our study shows the response of 'Rio Red' grapefruit leaves to the *E. australis* infection in terms of quantitative changes in the polyphenols. Total polyphenol levels decreased substantially in *E. australis*-infected 'Rio Red' grapefruit leaves 9 days post-inoculation. Levels of caffeic acid, naringin, naringenin, apigenin glucoside, luteolin glucoside and eriodictoyl decreased with *E. australis* infections indicating the pathogen's ability to overcome some of plant's defenses. Compared to 9-day controls, 9-day inoculated leaves had much less Eriodictoyl. Further studies are needed to fully understand mechanism of involvement of each of these polyphenol in the plant-pathogen interaction.

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